SURVIVAL OF ADHERING CORTICAL NEURONS ON POLYETHYLENIMINE MICROPATTERNS

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Abstract **The influence of neuron-adhesive pattern geometry on long-term survival of cortical neural tissue (rat brain) was studied over a time period of 15 days. Microwells (depth 0.5 μm) with diameters of 25, 50, 100 and 150 μm and inter-microwell distances of 15, 30, 60 and 90 μm, were etched in a neuron-repellent fluorocarbon (FC)-layer and coated with neuron-adhesive polyethylenimine (PEI). Results showed that the survival of neural tissue was geometry- independent after 1, 4 and 8 days but was favored on 150 μm wells after 15 days.**

Key words - **Cortical neurons, patterning, adhesion, polyethylenimine, fluorocarbon, viability, miniaturization**

I. INTRODUCTION

In neuroscience community, a growing interest is observed in the development of neuro-electronic devices that can survive in a physiological surrounding over prolonged periods of time. The development of a cultured neuron probe [1] is considered to be an important gateway towards improved integration of neural tissue with electronic devices, which is an advantage for effective and highly selective stimulation of nerve fibers and corresponding muscle functions. An essential part of a cultured probe is an efficient contact between electrode and neural tissue for sufficient electrical signal transfer [2]. Therefore we need to improve the neuron-adhesive character of electrodes and avoid neural adhesion around electrodes over longer periods of time. Photolithography [3-5] facilitates the preparation of chemical patterns of neuron-adhesive substances on micro-electrode devices [6] and is a promising tool towards accurate positioning of neurons on top of micro-electrodes.

The number of possible pattern geometries that can be prepared with photolithography methods are in fact endless. In neuroscience, typical examples of pattern geometries that have been investigated more frequently than others are patterns with multiple parallel tracks of adhesive/non-adhesive materials [7-9] and so-called grid patterns [3,10]. The choice for grid patterns is, amongst other reasons, driven by the desire to 1) control the positioning of adhering neurons onto specified locations on the surface and 2) maintain the possibility to obtain a connected two-dimensional neural network. Multi-electrode arrays (MEAs) are suitable instruments to investigate bioelectrical activity within such a network and are primarily used as tools to record electrical signals [11,12] although simultaneous stimulation and recording is a possible option [13]. Our group is interested in the application of MEAs for selective stimulation of sprouting axons developed by motor nerves. The final goal is an improved fine control of muscles. *In vitro* cultured islands of neurons on top of the MEAs are part of our strategy to attract axonal sprouts from nerves onto micro-electrodes. The miniaturization of MEAs into cultured probes with acceptable sizes raise questions about the optimal dimensions of the *in vitro* cultured islands of neurons, suitable for long-term adhesion and survival.

The aim of this paper is to study the adhesion and viability of dissociated cortical neurons on stepwise miniaturized chemical patterns over a time period of 15 days. Neurons were patterned with a combination of neuron-adhesive polyethylenimine (PEI) and a neuron-repellent fluorocarbon (FC) layer.

II. MATERIALS AND METHODS

Preparation procedure of chemical patterns with PEI and Plasma-FC using photolithography

Glass plates (Glaverbel, Mol, Belgium) were initially spin-coated with a layer of Polyimide (PI, Probimide 7510®, Arch Chemicals N.V., Zwijndrecht, Belgium) which was diluted in n-methyl pyrolidon (1:1 volume ratio). In a reactive ion etching (RIE) system, glass plates were covered with a Fluorocarbon (FC) layer using a low-energy CHF_3 plasma. Patterns with different geometries were etched in the FC-layer using spinned photoresist as a protective layer for the FC. CHF_3 exposed photoresist was removed by ultrasonic cleaning in acetone for 5 minutes. Positive photoresist was spinned onto the patterned surfaces (4000 rpm, 20 seconds) and the chromium UV-mask was accurately aligned (± 1 μm) with the visible topographical features of the pattern. Photoresist inside the circular wells was exposed to UV-light, developed and selectively removed (Developer OPD 4262, Arch Chemicals N.V., Zwijndrecht, Belgium). Glass plates were cut mechanically into 9 similar pieces (referred to as culture samples). Each culture sample contained 4 areas with different pattern geometries.

Culture samples were immersed in a PEI-solution (10 μg/ml) for a time period of 1 minute and subsequently immersed in Milli-Q water for 1 minute. Remaining photoresist with adsorbed PEI on top was selectively lifted-off with a 1.0 NaOH solution in a time-span of 2 minutes [14]. Removal of residual NaOH was done with a two-fold rinsing procedure in Milli-Q water for 1 minute.

Geometrical characteristics

The 4 pattern areas on each culture sample consisted of neuron-adhesive PEI-coated microwells, embedded in a

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neuron-repellent Plasma-FC layer. The distance between the microwells was kept at a constant fraction of 0.6 times the diameter D of the wells (see figure 2). The 4 patterns contained well diameters D of 150, 100, 50, and 25 µm. Consequently, this corresponded to separation distances of 90, 60, 30, and 15 µm between the wells. A total of 4 culture samples were tested.

Cortical neuron isolation and procedures:

Cerebral cortex from 1-day-old newborn rats was dissected out under sterile circumstances and dissociated with 0.25 % Trypsin/EDTA for 45 minutes in an incubator at 37 °C at 5 % CO_2 and subsequently treated with Soybean Trypsin Inhibitor (STI, 1 mg/ml) and Desoxyribonuclease I (DNAse I, 1.1 unit/ml). Trypan blue stain (0.4 %) was used to discriminate and count living neurons in a Bürker chamber, prior to the sedimentation of the neurons onto the culture samples. The plating density of living neurons was set at 2500 neurons/mm^2. Neurons were allowed to adhere onto the culture samples during a time period of 4 hours. The assembly of the 4 pattern geometries on each culture sample effectively reduced the amount of neural tissue required. In addition, the possibility of exposing the 4 patterns to neurons with unwanted variable adhesion ability (or viability) was avoided.

Viability staining

Viable and non-viable neural tissue was evaluated with a staining procedure using acridine orange (AO) and propidium iodide (PPI) respectively. Stock solutions of acridine orange (1000 µg/ml; Serva Feinbiochemica, Heidelberg, Germany) and propidium iodide (40 µg/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) were prepared in 0.1 M phosphate buffered saline (PBS). In a first run, tissue on micropatterned surfaces was stained with PPI to evaluate the presence of only non-viable tissue. Simultaneous staining of viable and non-viable tissue with both AO and PPI was done in a second run. In detail, each sample with adhering tissue was rinsed twice with fresh PBS and then immersed into a diluted PPI solution (5.0 µg/ml) for 5 minutes (first run). PPI stained (non-viable) tissue was examined with epi-fluorescence equipment mounted on an Nikon inverted microscope Diaphot-TMD (Nikon, Japan). Photomicrographs of fluorescent and phase contrast images were taken with a Nikon-FE 35 mm camera, attached to the front camera port. In the second run, the samples were immersed in a combined AO (1.5 µg/ml)/PPI (5.0 µg/ml) solution for 5 minutes. The 450-490 nm excitation filter in combination with a 510 nm dichroic mirror and 520 nm barrier filter permitted the simultaneous observation of both viable (green) and non-viable (red) tissue. Viability stains were done on day 1, 4, 8, and 15 days. The areas covered with red (non-viable) tissue were determined and referred to as $A_{NV}(D,T)$. The subsequent staining with acridine orange (AO) and propidium iodide (PPI) resulted in images with red, green and yellow areas. Assuming that yellow is the cumulative effect of non-

viable and viable cells overlapping each other, as was observed before for islets of Langerhans [15], the surface area where viable cells can be located is a summation of green and yellow areas and is referred to as $A_V(D,T)$. A time- and pattern-dependent viability factor V(D,T) was defined as the ratio of the area covered with viable neurons $A_V(D,T)$ with respect to the summation of areas covered with viable tissue $A_V(D,T)$ and non-viable tissue $A_{NV}(D,T)$

$$V(D,T) = \frac{A_V(D,T)}{A_V(D,T) + A_{NV}(D,T)} \qquad (1)$$

Quantitative analysis

Photomicrographs of stained neural tissue were scanned, stored as bitmap images, processed in Microsoft compatible software (Corel PhotoPaint 7, Corel Corporation, Ontario, Canada) until 1-bit images (black and white) were finally obtained. The images were stored as 8-bit images. Black and white corresponded to the empty background and the neural-tissue-covered foreground respectively. The software permitted the calculation of mean brightness values within 8-bit images (histogram function) and was used to calculate the total area of the surface covered with neural tissue (white: brightness value 255).

III. RESULTS

Fig. 1 demonstrates the calculated viability factor V(D,T) of neural tissue on 4 micropatterns over a time period of 15 days. The general observation was that the viability of
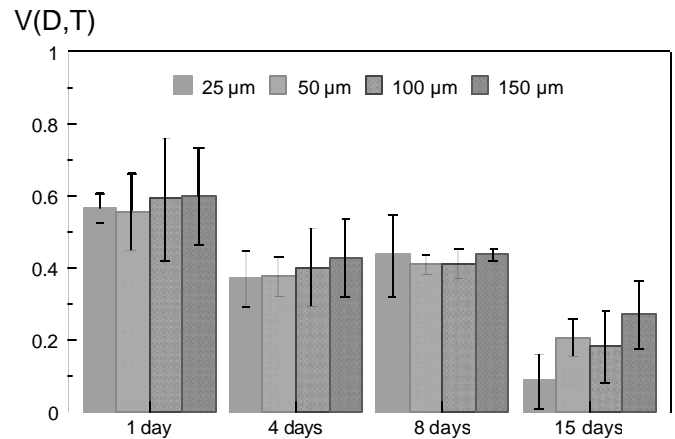

Fig.1. The viability factor V(D,T) on 4 micropatterns with corresponding well diameters D after T = 1, 4, 8 and 15 days. The mean ± SD over 4 images, collected from two tissue isolation experiments, is presented.

neural tissue decreased within this time-span and is rather insensitive to the underlying micropatterns after 1, 4, and 8 days. However significant differences in favor of the pattern with 150 µm wells became apparent after 15 days.

Fig. 2 presents phase contrast and fluorescence images of neural tissue after 15 days on 150 µm wells. Neural tissue was preferentially observed on the PEI-coated wells. Non-viable tissue was present after 15 days as was proven by the

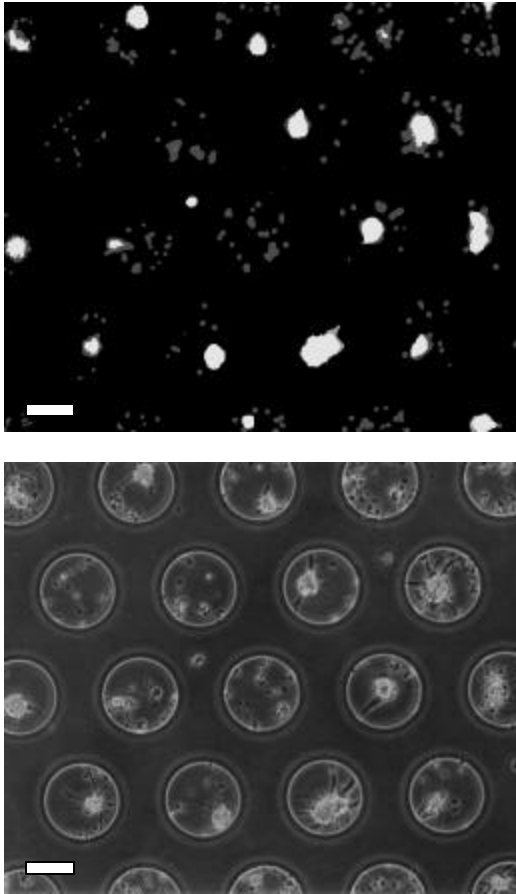red color after staining with propidium iodide (see Fig.2, Top). The images were predominantly red and yellow after

Fig.2. Fluorescent image (Top) and phase contrast image (Bottom) of AO/PI-stained neural tissue after 15 days on a pattern with 150 µm microwells. Scaling bars represent 100 µm.

simultaneous staining with propidium iodide and acridine orange (Fig.2, Bottom). The red color caused by propidium iodide staining was replaced by a yellow color and especially at locations where aggregates of neurons were observed. Only single isolated neurons kept their red color after simultaneous staining. Areas with a green color were rarely observed.

IV. DISCUSSION

Survival of tissue was relatively independent of pattern geometry in the first 8 days but favored the 150 µm wells after 15 days. This is explained by a more frequent detachment of aggregates from the smaller microwells. Since living tissue could primarily be traced as part of an aggregate, the enhanced detachment of aggregates from the patterns with smaller microwells effectively removed most living tissue. The non-adhesive FC, underlying the aggregates, accounts for the enhanced detachment on small wells as opposed to the described events on 150 µm wells where aggregates adhere on PEI only. A general observation on all

patterns is a decreasing viability of the neural tissue over 15 days. Authors have occasionally reported more viable
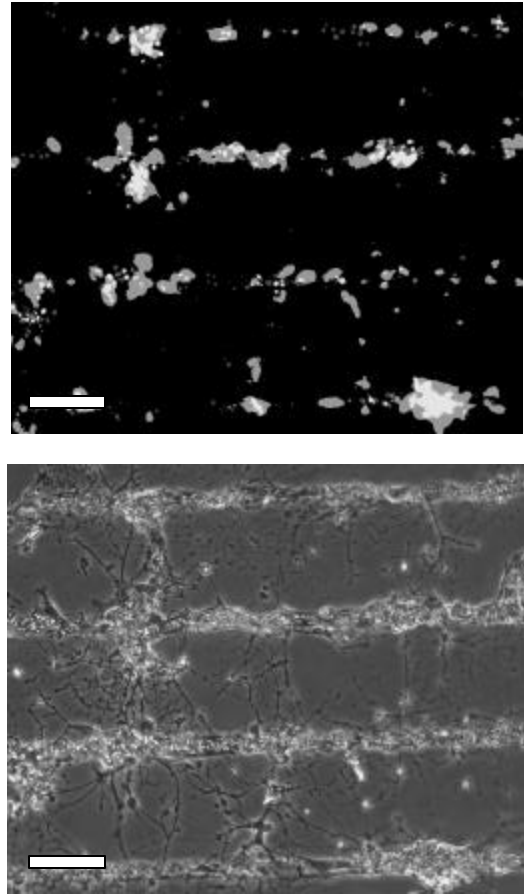

Fig.3. Fluorescent image (Top) and phase contrast image (Bottom) of AO/PI-stained neural tissue after 15 days on a micro-printed pattern with 24 µm wide multiple parallel tracks. Heart-to-heart distance between tracks is 160 µm. Scaling bars represent 100 µm.

patterning systems with hippocampal neurons on micro-stamped poly-lysine grid patterns [16]. Although results are always difficult to compare due to differences in the experimental set-up, it could be hypothesized that the inherent connectivity and/or adhesion of microprinted grid patterns stabilizes the network and renders to be more suitable for long-term survival. Therefore microcontact printed patterns using PEI as the printing substance and the FC-layer as the background material (Fig.3) were also prepared and tested for their survival. Indeed, it was observed that calculated viability factors of double-stained patterns with multiple parallel tracks were around 0.8 and well above the numbers found for microwells with different dimensions (see Fig.1).

V. CONCLUSION

The conclusion is that moderate instead of advanced miniaturization of a neural pattern with isolated islands of neurons will slow down the necrosis and detachment of neural tissue. The underlying mechanisms probably involve

the secretion and diffusion of endogenous proteins, which depend upon the concentration gradients and distances between the neurons. Furthermore, necrosis of neural tissue is likely to be triggered by a hampered diffusion of medium nutrients and endogenous proteins towards the center of neuron aggregates [17]. Apparently, the geometrical characteristics of our most miniaturized patterns (25 µm wells) are unsuitable for diffusion-assisted long-term adhesion and survival of neural tissue. This is unfortunate because miniaturization towards single-cell size dimensions would allow a more precise positioning of cells onto small pre-defined areas [4] such as microelectrodes [18,19]. Survival of cortical neurons is favored on microwells with a diameter of 150 µm. Microcontact printing of PEI onto a Fluorocarbon layer appears to be a promising method to improve the long-term survival of adhering neural tissue islands.

REFERENCES

[1] J. Pine, M. Maher, S. Potter, Y-C Tai, S. Tatic-Lucic, and J. Wright, "A cultured neuron probe," *Proc. Int. Conf. of the IEEE Eng. In Med. & Biol. Soc.* CD-Rom, 1996.

[2] D. Kleinfeld, K.H. Kahler, and P.E. Hockberger, "Controlled outgrowth of dissociated neurons on patterned substrates," *J. Neurosci.*, vol. 8, pp. 4098-4120, 1988.

[3] J.M. Corey, B.C. Wheeler, and G.J. Brewer, "Micrometer resolution silane-based patterning of hippocampal neurons: critical variables in photoresist and laser ablation processes for substrate fabrication," *IEEE Trans. Biomed. Eng.*, vol. 43, pp. 944-955, 1996.

[4] R. Singhvi, A. Kumar, G.P. Lopez, G.N. Stephanopoulos, D.I.C. Wang, G.M. Whitesides, and D.E. Ingber, "Engineering cell shape and function," *Science*, vol. 264, pp. 696-698, 1994.

[5] J.C. Chang, B.C. Wheeler, and G.J. Brewer, "Recording of patterned neural networks," *Proc. BMES/EMBC Conf. Atlanta*, pp. 362, 1999, CD-ROM.

[6] D.V. Nicolau, T. Taguchi, H. Taniguchi, H. Tanigawa, S. Yoshikawa, "Patterning neuronal and glia cells on light-assisted functionalised photoresists," *Biosensors & Bioelectronics*, vol. 14, pp. 317-325, 1999.

[7] A. Ohl, K. Schroder, D. Keller, A. Meyer-Plath, "Chemical micropatterning of polymeric cell culture substrates using low-pressure hydrogen gas discharge plasmas", *J. Mater.Sci:Mater in Medicine*, vol. 10, pp. 747-754, 1999.

[8] C.L. Klein, M. Scholl, A. Maelicke, "Neuronal networks *in vitro*: formation and organization on biofunctionalized surfaces", *J. Mater.Sci:Mater in Medicine*, vol. 10, pp. 721-727, 1999.

[9] M.Matsuzawa, S.Tokumitsu, W.Knoll, and H.Sasabe, Effects of organosilane monolayer films on the geometrical guidance of CNS neurons, *Langmuir*, vol. 14, pp. 5133-5138, 1998.

[10] H.G. Craighead, S.W. Turner, R.C. Davis, C. James, A.M. Perez, P.M. St.John, M.S. Isaacson, L. Kam, W. Shain, J.N. Turner, and G. Banker, "Chemical and topographical surface modification for control of central nervous system cell adhesion", *J. Biomed. Microdev.*, vol. 1:1, pp. 49-64, 1998.

[11] C.A. Thomas, P.A. Springer, G.E. Loeb, Y. Berwald-Netter, and L.M. Okun, "A miniature microelectrode array to monitor the bioelectric activity of cultured cells", *Exp. Cell. Res.*,74 , pp. 61-66, 1972.

[12] G.W. Gross, E. Rieske, G.W. Kreutzberg, and A. Meyer, "A new fixed-array multielectrode system designed for long-term recording of extracellular single unit activity in vitro", *Neurosci. Lett.*, vol. 6, pp. 101-105, 1977.

[13] M.P. Maher, J. Pine, J. Wright, "The neurochip: a new multielectrode device for stimulating and recording from cultured neurons, *J. Neurosci.Methods*, vol. 87, pp. 45-46, 1999.

[14] T.G.Ruardij, M.H.Goedbloed, and W.L.C. Rutten, "Adhesion and patterning of cortical neurons on Polyethylenimine- and Fluorocarbon-coated surfaces", *IEEE Trans. Biomed. Eng.*, vol. 47, pp. 1593-1599, 2000.

[15] H.L. Bank, "Rapid assesment of islet viability with acridine orange and propidium iodide", *In Vitro Cell.& Dev. Biology*, vol. 24, pp. 266-273, 1988.

[16] D. W. Branch, B. C. Wheeler, G. J. Brewer, and D. E. Leckband "Long-Term Maintenance of Patterns of Hippocampal Pyramidal Cells on Substrates of Polyethylene Glycol and Microstamped Polylysine", *IEEE Trans. Biomed. Eng.,* vol. 47, pp. 290-300, March 2000.

[17] G.J. Harry, M. Billingsley, A. Bruinink, I.L. Campbell, W. Classen, D.C. Dorman, C. Galli, D.Ray, R.A. Smith, H.A. Tilson, "In vitro techniques for the assessment of neurotoxicity", *Environmental Health Perspectives*, vol. 106 (suppl. 1), pp.131-158, 1998.

[18] J.U. Meyer, M. Schüttler, H.Thielecke, and T. Stieglitz, "Biomedical microdevices for neural interfaces", *Proceedings of the 1st annual international IEEE-EMBS special topic conference on microtechnologies in medicine & biology*, pp.447-453, Lyon October 2000.

[19] J.R. Buitenweg, W.L.C. Rutten, W.P.A. Willems, and J.W. van Nieuwkasteele, "Measurement of sealing resistance of cell-electrode interfaces in neuronal cultures impedance spectroscopy", *Medical & Biological Engineering & Computing*, vol.36, pp.630-637, 1998.